The Role of CXCR3 in Breast Cancer Tumorigenesis caused by PyMT Cell Line

Presented in Partial Fulfillment of the Requirements for graduation

"With Research Distinction in Microbiology" in the Undergraduate

Colleges of The Ohio State University

by

Charles Penvose

The Ohio State University

June 2012

Project Advisor: Professor Abhay Satoskar, Department of Microbiology

ACKNOWLEDGEMENTS

I would like to thank Dr. Abhay Satoskar for his continuous facilitation, support, and excellent direction throughout my thesis experience. Dr. Satoskar's mentorship and personal example always helped me strive and push myself to the next level. I would also like to thank Dr. Steve Oghumu for his support and advice throughout my thesis work. Finally, I would like to thank the Office of Undergraduate Studies & Research for funding my research and the entire faculty of the Department of Microbiology for their support.

The Role of CXCR3 in Breast Cancer Tumorigenesis caused by PyMT Cell Line

Abstract:

Breast cancer is responsible for the highest number of cancer related deaths in women each year. Recent breast cancer patient studies have shown that tumor cell expression of CXCR3, a chemokine receptor, results in poor overall survival. Conversely, another study shows that expression of CXCR3 on activated CD8 T cells enable migration to cancer sites, thereby contributing to tumor rejection. The purpose of this research is to determine whether or not CXCR3 plays a role in the tumorigenesis in a PyMT breast cancer mouse model. We examined the role of CXCR3 in breast cancer in vivo by infecting female CXCR3-/- and wild type mice with a Polyoma Middle T antigen (PyMT) breast cancer cell line. The immune response to the induction of tumorigenesis was examined through tumor measurements and FACS analysis of mouse tumors, spleens and lymph nodes. Initial findings indicate a significantly enhanced tumor growth in knockout mice, with knockout mice displaying tumor volumes three orders of magnitude greater than wild type mice. Further, the spleens and lymph nodes of CXCR3-/- mice contained lower numbers of infiltrating CD8+ T cells and CD4+ T cells and showed elevated numbers of alternatively activated macrophages (M2). In conclusion, our results demonstrate that CXCR3 plays a role in the immune response to tumorigenesis in a PyMT breast cancer mouse model.

Introduction:

Breast cancer is a devastating disease responsible for many deaths of men and women throughout the nation. According to U.S. Breast Cancer Statistics, breast cancer death rates for women are higher than any other cancer besides lung cancer. Additionally, about 1 in 8 women will develop breast cancer in her lifetime (3). The immune system plays a major role in responding to cancer. Immune cells aggregate to the site of the tumor to defend against the tumor development.

Research has shown that communication among various immune cells via chemokines is essential for leukocyte migration, metastasis, angiogenesis, and tumorigenesis (1). The CXCR3 chemokine receptor has been shown to be associated with many types of cancer including breast cancer. CXCR3 is activated by three (IFN)-γ inducible ligands CXCL9, CXCL10, and CXCL11 (2). CXCR3 is induced on naïve T lymphocytes after activation and is highly expressed on Th1 associated T Helper lymphocytes, CD8 lymphocytes. Also, CXCR3 is present on cells of the innate immunity such as Natural Killer (NK), and Natural Killer T cells (NKT) (5). CXCR3 facilitates the migration of Th1 helper T cells and CD8 T cells to the tumor microenvironment allowing the lymphocytes to interact with antigen presenting cells, resulting in more effecter cells and memory cells (5). The Th1 response is known to suppress the tumorigenesis whereas the Th2 response creates an environment permissive for tumorigenesis (5).

While some research suggests that CXCR3 is beneficial when expressed on immune cells, other research shows that CXCR3 is expressed on tumor cells leading to poor prognosis. CRCX3 promotes the growth of human glioblastoma multiforme (4). Additionally, Studies have shown

CXCR3 is expressed by tumor cells during melanoma, ovarian carcinoma, renal carcinoma, and breast cancer. (4). CXCR3 expression on cancer cells is correlated with poor patient prognosis. This indicates that CXCR3 has opposing effects when expressed on cells of the tumor microenvironment and when expressed on tumor cells.

Little research has been published on the relationship between CXCR3 and breast cancer. Most studies focus on the metastasis of tumor cells expressing CXCR3 and use patient studies (10, 16). The focus of this research is to investigate the role of CXCR3 on lymphocytes, macrophages, and natural killer cells in breast cancer in vivo using a murine model. We hypothesize that CXCR3 is necessary for tumor suppression, and that knocking out CXCR3 will result in larger tumors in CXCR3 knockout mice than wild type mice.

Materials and Methods:

PyMT Cell Line:

The PyMT cell line was used to induce breast cancer in the mice. According to studies, the PyMT provides a realistic model of human breast cancer (6). The PyMT are comparable to human breast cancer in morphology and have four distinctly identifiable stages of tumor progression similar to human breast cancer. In addition, the expression of biomarkers in PyMT-induced tumors is also consistent with those associated with poor outcome in humans. (6)

Mice/Infection:

CXCR3 -/- and wild type female C57BL/6 mice were injected with 1 X 106/100 uL PyMT cells in their mammary gland. Sample mice included 5 WT and 5 CXCR3 -/- mice for Experiment 1 8 KO CXCR3 mice and 13 WT mice injected for Experiment 2.

Tumor Measurements:

Tumor volumes were measured every other day once the initial tumor developed. Tumor volume was calculated using the formula V = 0.52 X a2 X b, where (a) corresponds with the smallest superficial tumor diameter and (b) corresponds with the largest superficial tumor diameter. Mice were sacrificed after 62 days.

FACS Analysis:

Fluorescence-activated cell-sorting (FACS) analysis was performed on single cell suspensions derived from tumor, spleen, and lymph node tissue harvested from every mouse injected with PyMT cells. The spleen and lymph nodes were examined because they play a major role in the immune system by filtering body fluid of foreign molecules. For macrophage specific staining, the single cell suspensions were incubated with anti-F4/80, anti-mannose receptor, anti-CD-11b, and anti-IL-4 receptor alpha. For lymphocyte staining, cells were incubated with anti-CD3, anti-CD4, anti-CD8, and anti-NK1.1. Stained cells were analyzed with a FACS Caliber machine and results were analyzed with FlowJo software.

Results: Average Tumor Volumes

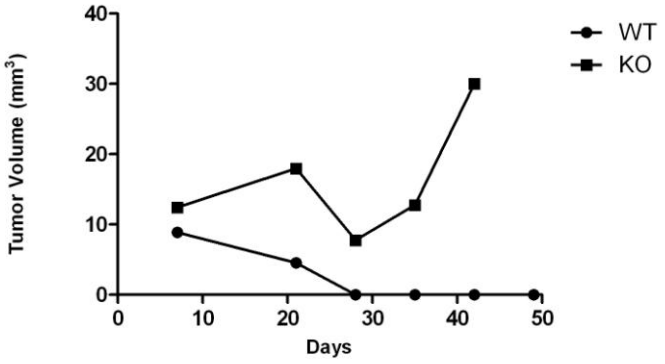

Figure 1A: The average tumor volumes for Experiment 1.

The tumor volumes for experiment one were taken one week post infection and continued until day 49 when mice had to be sacrificed because tumor size exceeded the maximum volume regulation of 2 cm^3. Superficial tumors were observed 7 days after injection for both wild type and CXCR3 KO mice however; the volumes decreased at week 4 for all of the wild type mice. One CXCR3 KO mouse continued growing a tumor until week 7 when the tumor became greater than the maximum volume regulation. The average tumor volumes of the KO mice were observed to have larger average tumor volumes than the average tumor volumes of the WT mice.

Results: Average Tumor Volumes

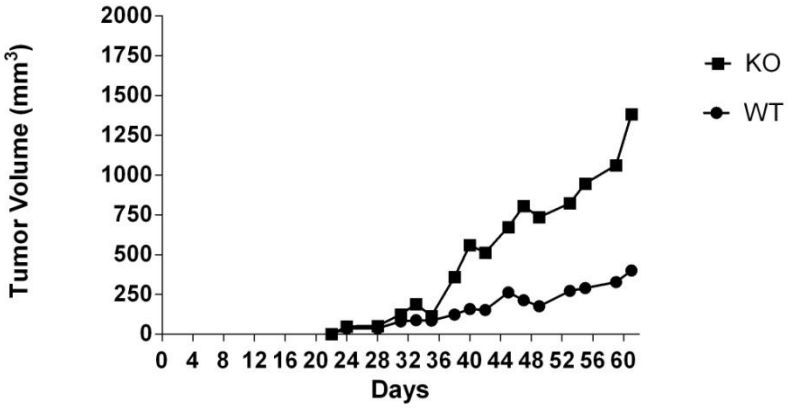

Figure 1B: The average tumor volumes for Experiment 2. Samples included. The tumors developed

During Experiment 2 the knockout mice started to differentiate from the wild type mice at four weeks post-injection. The initial measurements are only available three weeks post-injection due to tumor visibility. There were 7 WT mice with tumors and 4 CXCR3 KO mice with tumors that were measured until the end point at 62 days post infection. At 62 days tumors began necrotizing and exceeded the maximum regulation volume. Similarly to Experiment 1, the average tumor volumes for the CXCR3 KO mice were larger than the average tumor volumes for the WT mice.

Results: FACS Data Experiment 2

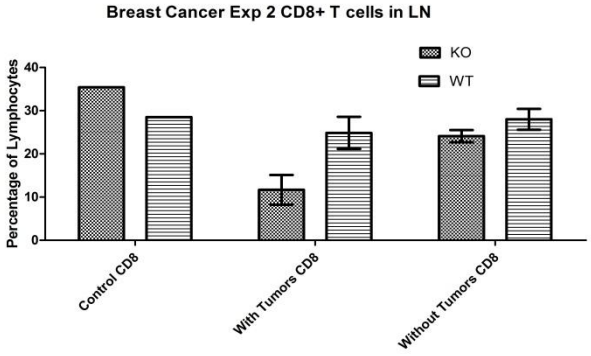

Figure 2A. Percentage of CD4+ T cells in the lymph nodes of KO and WT mice 62 days after PyMT cell injection. Control mice were not injected withPyMT cells. These percentages are from FACS analysis with gating on CD3+ and then CD4+, CD8– cells.

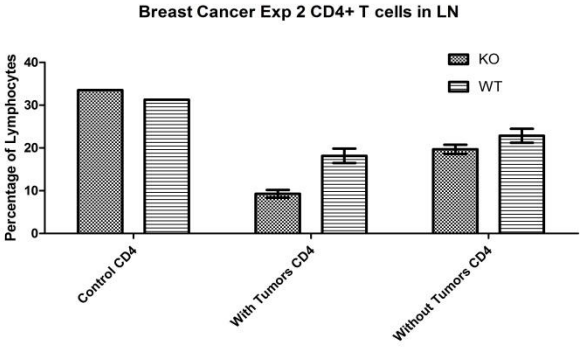

Figure 2B. Percentage of CD8+ T cells in the lymph nodes of KO and WT mice 62 days after PyMT cell injection. Control mice were not injected with PyMT cells. These percentages are from FACS analysis with gating on CD3+ and then CD8+, CD4– cells.

The FACS results for Experiment 2 examine the immune cells of the lymph nodes quantitatively. Figures 2A and 2B show the percentage of the lymphocytes that are present in the Lymph nodes of the mice. The Decrease in CD4 T lymphocytes shown Figure 2B between the CXCR3 KO and WT is statistically significant with $P<0.05$. There is also an observable decrease in the percentage of CD8 T lymphocytes however; this difference is not statistically significant.

Results: FACS Data Experiment 2

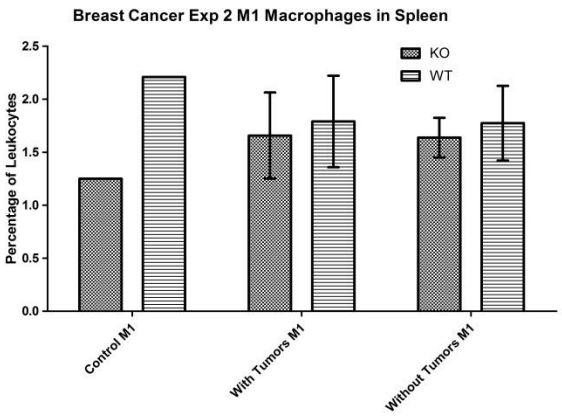

Figure 3A. The percentage of M1 macrophages in the spleen of KO CXCR3 and WT mice 62 days after PyMT cell injection. Control mice were not injected with PyMT cells. These percentages were determined from gating on F4/80+, Mannose Receptor - cells.

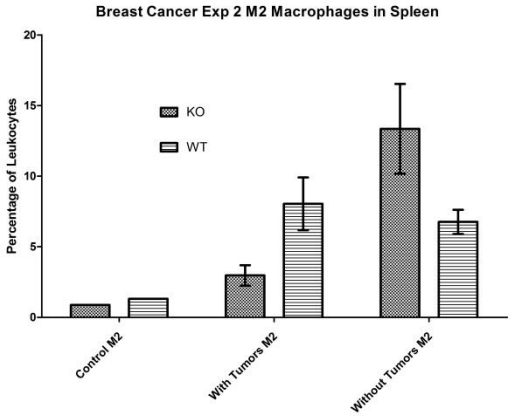

Figure 3B. The percentage of M2 macrophages in the spleen of KO and WT mice 62 days after PyMT cell injection. Control mice were not injected with PyMT cells. These percentages were determined from gating on F4/80+, Mannose Receptor+ cells.

The FACS results for Experiment 2 examine the immune cells of the spleen quantitatively. Figures 3A and 3B show the percentage of the macrophages that are present in the spleens of the mice. The decrease in M2 alternatively activated macrophages in the CXCR3 KO mice compared to the WT mice is statistically significant with $P<0.05$. There was no observable change in the classically activated M1 macrophages between the WT and CXCR3 KO groups.

Discussion:

The average tumor volumes and FACS analysis provide interesting clues as to CXCR3's role in breast cancer tumorigenesis. CXCR3 knockout mice had lower average tumor volumes than the WT mice which was consistent with the initial hypothesis. The differences in tumorigenesis of the breast cancer are dependent on the type and action of immune cells present in the tumor environment. These cells create tumor environments by honing in immune effector cells via chemokines, secreting cytokines to induce a pro-inflammatory response, or honing in specific lymphocytes to suppress the pro-inflammatory immune response.

FACS analysis indicated that there was a fluctuation in the lymphocyte populations in the lymph nodes between the groups. CXCR3 forms an inflammatory chemokine system capable of coordinating pro-inflammatory T lymphocyte responses in the periphery, making Th1 lymphocytes an integral part of the acquired anti-tumor immune response (5). Results showed that KO mice had significantly lower CD4+ lymphocytes than the WT mice. The T helper lymphocytes also induce anti-tumor effects by coordinating the pro-inflammatory immune response (5). The decrease in the CD4+ lymphocytes in the CXCR3 KO mice could contribute to the observed increased tumor volume.

Although not statistically relevant, FACS analysis data also showed that more CD8+ lymphocytes were present in WT mice lymph nodes compared to KO mice lymph nodes. According to patient studies of CD8+ lymphocytes in breast cancer, the more CD8+ T lymphocytes surrounding the tumor correlated to increased breast cancer survival (8,10). The

surrounding CD8+ cells are vital to the inflammation response and therefore anti-tumor immunity. CD8+ T cells can differentiate to become cytotoxic lymphocytes (CTLs), which secrete IFNγ along with other effector molecules providing a Th1 immune response (5). The CXCR3 is correlated to the Th1 type differentiation of lymphocytes. Furthermore, research has shown that CXCR3 is required on CD8+ cells for infiltration into the brain during cerebral malaria (9). CXCR3 KO mice were protected from cerebral malaria due to reduced CD8+ CTL entry into the brain. The CXCR3 is necessary for the CD8+ to gain entry into sites of inflammation (9).

Reduced CD8+ lymphocytes may be contributing to the increased tumor volumes of the CXCR3 KO mice in the PyMT model of breast cancer. The CXCR3 may be necessary for honing in the CD8+ lymphocytes. Investigation of the number of CD8+ lymphocytes in the tumor and surrounding the tumor would be necessary to make more solid conclusions. The Th1 response is also regulated by the activation of macrophages present in the tumor microenvironment.

Macrophages secrete cytokines and educate naive T lymphocytes into different effector T lymphocytes. We investigated the spleen to determine if the amount of macrophages fluctuated between the WT and CXCR3 KO mice. FACS analysis of macrophages shows that there was no fluctuation in the number of classically activated M1 macrophages in the spleen between the two groups of mice. This suggests that a Th1 pro-inflammatory response is occurring in both WT and CXCR3 KO mice. Th1 cytokines can trigger the formation of classically activated or M1 macrophages (11). The amount of M2's fluctuated between groups.

The number of M2 alternatively activated macrophages decreased in the CXCR3 KO mice without tumors compared to the WT mice however; The CXCR3 KO mice with tumors had an increase of M2 macrophages. The M2 are induced by cytokines such as: IL-4, IL-10, TGF-β

and M-CSF (11). The M2 macrophages induce anti-inflammatory immune responses. Tumor-associated macrophages have been shown to have M2 anti-inflammatory phenotypes (11). In human breast cancer, an increased amount of tumor-associated macrophage (TAM) correlates with poor patient prognosis. In mouse models of breast cancer, eliminating TAM's from the tumor site results in less tumor progression (11). An increase in M2 may be contributing to larger tumors due to an anti-inflammatory effect on the tumor microenvironment. Results are contradictory between the mice with tumors and without tumors in the groups. Several mice from WT and KO groups were found to contain tumors in the peritoneal cavity making their tumor measurements impossible. Future studies will use isoflurane to anesthetize the mice creating a more uniform injection. More experiments will need to be conducted in order to make more conclusions concerning the M2 macrophage fluctuations between groups.

In addition to improvements to future experiments, investigation into other subgroups of lymphocytes, tumor-induced myeloid –derived suppressor cell populations, and natural killer cells may provide further insight into role of CXCR3 in human breast cancer. Studies show that a decrease in CD4+CD25+ Regulatory T cells (T Regs) enhances cell-mediated anti-tumor immunity (15). Also, the depletion of T Regs was shown to stimulate the NK T cells (15). Research into the NK cells' role in anti-tumor immunity has been performed with human breast cancer. Finding indicate that the dysfunction of NK cells results in human breast tumor progression (16). Lastly, research into myeloid-derived suppressor cell populations has provided data suggesting they are an important for the immune-evading mechanism used by tumors. These are heterogeneous populations of cells with diverse roles however; many have been shown to suppress T lymphocytes (17).

In conclusion, results indicate that CXCR3 plays a definite role in the tumorigenesis of breast cancer in the PyMT mouse model however; investigations into different anti-tumor associated immune cells and additional experimental trials are necessary for a clearer view of this role. Real-time PCR, along with western blots, may be helpful in determining what tumor related proteins are being produced and the various pathways of the immune cells. The levels of proteins involved in angiogenesis, metastisis, and tumorigenesis may all be interesting in explaining the difference in tumor sizes between the CXCR3 KO and WT mice. Future research may lead to therapeutic advances in the clinical treatment of human breast cancer.

References:

1. Vandercappellen, J., Vandamme, J., & Struyf, S. (2008). The role of CXC chemokines and their receptors in cancer. Cancer Letters, 267(2), 226-244. doi: 10.1016/j.canlet.2008.04.050
2. Groom JR, Luster AD. CXCR3 ligands: redundant, collaborative and antagonistic functions. Immunology and Cell Biology. 2011; 89(2):207–215. [PubMed]
3. BreastCancer.org - Breast Cancer Treatment Information and Pictures. (n.d.). BreastCancer.org - Breast Cancer Treatment Information and Pictures. Retrieved from http://www.breastcancer.org/
4. Liu C, Luo D, Reynolds BA, Meher G, Katritzky AR, Lu B, Gerard CJ, Bhadha CP, Harrison JK. Chemokine receptor CXCR3 promotes growth of glioma. Carcinogenesis. 2011 Feb;32(2):129-37. Epub 2010 Nov 3. [PubMed]
5. Groom JR, Luster AD. CXCR3 in T cell function. Exp Cell Res. 2011 Mar 10;317(5):620-31. [PubMed].
6. Lin EY, Jones JG, Li P. et al. Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. Am J Pathol. 2003;163:2113–26.
7. Walser TC, Rifat S, Ma X, Kundu N, Ward C, Goloubeva O, Johnson MG, Medina JC, Collins TL, Fulton AM. Antagonism of CXCR3 inhibits lung metastasis in a murine model of metastatic breast cancer. Cancer Res. 2006;66:7701–7. doi: 10.1158/0008-5472.CAN-06-0709.

8. Liu F, Lang R, Zhao J, Zhang X, Pringle GA, Fan Y, Yin D, Gu F, Yao Z, Fu L. CD8(+) cytotoxic T cell and FOXP3(+) regulatory T cell infiltration in relation to breast cancer survival and molecular subtypes. Breast Cancer Res Treat. 2011. [PubMed]

9. G.S. Campanella, A.M. Tager, J.K. El Khoury, S.Y. Thomas, T.A. Abrazinski, L.A. Manice, R.A. Colvin, A.D. Luster, Chemokine Receptor CXCR3 and its ligands CXCL9 and CXCL10 are required for the development of murine cerebral malaria, Proc. Natl Acad. Sci. USA 105 (2008) 4814–4819.

10. Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AH, Ellis IO, Green AR. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. J Clin Oncol. 2011;29(15):1949–1955. doi: 10.1200/JCO.2010.30.5037.

11. Laoui D, Movahedi K, Van Overmeire E, Van den Bossche J, Schouppe E, Mommer C, Nikolaou A, Morias Y, De Baetselier P, Van Ginderachter JA. Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions. Int J Dev Biol. 2011;55(7-9):861-7.

12. Lawrence T. Macrophages and NF-κB in cancer. Curr Top Microbiol Immunol. 2011;349:171-84.

13. Rebecca Liu and Weiping Zou Huang, Emily Finlayson, Diane Simeone, Theodore H. Welling, Alfred Chang, George Coukos. Relevance of Th17 cells in the Human Tumor Environments. Blood. 2009 114: 1141-1149 doi:10.1182/blood-2009-03-208249

14. Ilona Kryczek, Mousumi Banerjee, Pui Cheng, Linhua Vatan, Wojciech Szeliga, Shuang Wei, Emina J Wang, D Cai, B Ma, G Wu AND J Wu. Skewing the Balance of Regulatory T-cells and T-helper 17 Cells in Breast Cancer Patients. The Journal of International Medical Research 2011; 39: 691 – 701.

15. Hong H, Gu Y, Zhang H, et al. Depletion of CD4+CD25+ regulatory T cells enhances natural killer T cell-mediated anti-tumour immunity in a murine mammary breast cancer model. Clin Exp Immunol. 2010;159:93–9.

16. Mamessier E, Sylvain A, Thibult ML, Houvenaeghel G, Jacquemier J, Castellano R, Gonçalves A, André P, Romagné F, Thibault G, Viens P, Birnbaum D, Bertucci F, Moretta A, Olive D. Human breast cancer cells enhance self-tolerance by promoting evasion from NK cell antitumor immunity. J Clin Invest. 2011 Sep;121(9):3609-22. doi: 10.1172/JCI45816. Epub 2011 Aug 15

17. Movahedi K, Guilliams M, Van den Bossche J, Van den Bergh R, Gysemans C, et al. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. Blood. 2008;111:4233–4244.